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The twin-arginine translocation (Tat) systems from *Bacillus subtilis* display a conserved mode of complex organization and similar substrate recognition requirements

James P. Barnett¹, René van der Ploeg², Robyn T. Eijlander³, Anja Nenninger¹, Sharon Mendel¹, Rense Rozeboom², Oscar P. Kuipers³, Jan Maarten van Dijk² and Colin Robinson¹

¹ Department of Biological Sciences, University of Warwick, Coventry, UK

² Department of Medical Microbiology, University Medical Centre Groningen and University of Groningen, The Netherlands

³ Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Haren, The Netherlands

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Correspondence

C. Robinson, Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK
Fax: +44 (0)2476 523568
Tel: +44 (0)2476 523557
E-mail: colin.robinson@warwick.ac.uk
Website: <http://www2.warwick.ac.uk/fac/sci/bio/>

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The twin arginine translocation (Tat) system transports folded proteins across the bacterial plasma membrane. In Gram-negative bacteria, membrane-bound TatABC subunits are all essential for activity, whereas Gram-positive bacteria usually contain only TatAC subunits. In *Bacillus subtilis*, two TatAC-type systems, TatAdCd and TatAyCy, operate in parallel with different substrate specificities. Here, we show that they recognize similar signal peptide determinants. Both systems translocate green fluorescent protein fused to three distinct *Escherichia coli* Tat signal peptides, namely DmsA, AmiA and MdoD, and mutagenesis of the DmsA signal peptide confirmed that both Tat pathways recognize similar targeting determinants within Tat signals. Although another *E. coli* Tat substrate, trimethylamine *N*-oxide reductase, was translocated by TatAdCd but not by TatAyCy, we conclude that these systems are not predisposed to recognize only specific Tat signal peptides, as suggested by their narrow substrate specificities in *B. subtilis*. We also analysed complexes involved in the second Tat pathway in *B. subtilis*, TatAyCy. This revealed a discrete TatAyCy complex together with a separate, homogeneous, ~ 200 kDa TatAy complex. The latter complex differs significantly from the corresponding *E. coli* TatA complexes, pointing to major structural differences between Tat complexes from Gram-negative and Gram-positive organisms. Like TatAd, TatAy is also detectable in the form of massive cytosolic complexes.

The twin-arginine translocation (Tat) pathway operates in the bacterial plasma membrane where it serves to transport fully folded proteins into or across the membrane. This process is energized primarily, if not solely, by the proton motive force [1–4], and the Tat pathway functions alongside the well-characterized Sec pathway which translocates proteins in an unfolded conforma-

tion by an ATP-dependent mechanism. It appears that the Tat pathway exists to facilitate the transport of proteins that fold too tightly or rapidly in the cytosol to be compatible with the Sec pathway. It is also used to translocate proteins that require a cofactor to be inserted in the cytosol prior to transport, such as complex redox enzymes involved in the respiratory chain.

Abbreviations

GFP, green fluorescent protein; HRP, horseradish peroxidase; Tat, twin-arginine translocation; TMAO, trimethylamine *N*-oxide; TorA, trimethylamine *N*-oxide reductase.

Proteins are targeted to the Tat pathway by means of cleavable N-terminal signal sequences that contain a highly conserved twin-arginine motif within the consensus sequence (S/T-R-R-x-F-L-K) [5–7]. At least three distinct targeting determinants within this motif have been shown to be important for Tat translocation in bacteria [8].

Gram-negative bacteria contain three essential Tat components, namely the integral membrane proteins TatA, TatB and TatC. These have molecular masses of 10, 18 and 30 kDa, respectively, in *Escherichia coli* (which is by far the best studied bacterial Tat system). The genes encoding these three proteins are coexpressed in an operon with a fourth *tat* gene, *tatD*, which is not involved in the Tat pathway [9]. A fifth *tat* gene, *tatE*, is also present in *E. coli* and is expressed elsewhere in the genome. This gene is thought to be a cryptic gene duplication of *tatA* as it can functionally complement a *tatA* null mutant. The *tatE* gene is expressed at a very low level relative to the *tatA* gene and is not thought to play any significant role in the Tat pathway [10,11].

The three essential Tat components form two types of complexes within the plasma membrane: a substrate-binding TatABC complex of ~ 370 kDa, in which TatB and TatC are the critical components, and a series of separate TatA complexes that vary in size from < 100 kDa to well over 500 kDa [12,13]. It has been suggested that these TatA complexes are involved in the formation of pores through which Tat substrates are translocated [14], with the size variation perhaps linked to the need to transport substrates of differing size. Recently, some doubt has been cast on the functional significance of the size variation of the TatA oligomers, because mutant TatA proteins form such oligomers even in the absence of Tat-specific protein translocation [15].

The Tat systems of Gram-positive bacteria exhibit interesting differences to those of Gram-negative bacteria, the most striking of which is the absence of a TatB component in virtually all species. Some Gram-positive bacteria, such as *Bacillus subtilis*, also contain multiple Tat pathways that operate in parallel with differing substrate specificities [16]. *B. subtilis* is a harmless soil-dwelling bacterium that contains three *tatA* genes, denoted *tatAd*, *tatAy* and *tatAc*, and two *tatC* genes denoted *tatCd* and *tatCy*. The *tatAd* gene is expressed in an operon with *tatCd* and these two components form a minimal Tat translocase responsible for the translocation of the substrate PhoD. The *phoD* gene is expressed upstream of the *tatAd/Cd* genes and this operon is expressed under phosphate-limited conditions. PhoD is the only known substrate of the

TatAdCd system [17–19]. The protein has phosphodiesterase and alkaline phosphatase activity, and PhoD is targeted to the cell wall, where it is involved in the release of inorganic phosphate [20].

The absence of a TatB component led to the idea that the TatAd protein may be bifunctional, fulfilling the roles of both TatA and TatB of *E. coli* [16]. We confirmed this in a recent study by showing that TatAd could indeed complement both the *E. coli* *tatA/E* and *tatB* null mutant strains [21]. The TatAd and TatCd proteins were also shown to form two types of complexes within the membrane: a TatAdCd complex that is significantly smaller than its *E. coli* counterpart (~ 230 kDa as judged by Blue-native PAGE) and a homogeneous TatAd complex (~ 160 kDa as judged by gel filtration) that does not exhibit the same size variation as *E. coli* TatA complexes [21].

The *tatAy* and *tatCy* genes are coexpressed in an operon to form a second minimal Tat translocation pathway in *B. subtilis* [17]. This operon is constitutively expressed and only a single substrate has been identified for this pathway: YwbN, a heme-containing DyP-type peroxidase. The third *tatA* gene of *B. subtilis*, *tatAc*, is not expressed with any other Tat components and its contribution to the Tat pathway is not known [17,18,22]. Recently, two other *B. subtilis* Tat substrates (QcrA and YkuE) have been identified using a facile reporter system, although their preferred Tat pathway for secretion in *B. subtilis* is not yet known [23].

In this study, we investigated the substrate specificities of the two Tat pathways of *B. subtilis* in order to determine whether they are predisposed to recognize specific Tat signals. We show that both the TatAdCd and TatAyCy systems recognize surprisingly similar targeting determinants despite their distinct substrate specificities within *B. subtilis*. In addition, we show that, like TatAdCd, the TatAyCy system consists of two types of complexes within the membrane, a TatAyCy complex and a separate TatAy complex that resemble more closely the TatAdCd and TatAd complexes than the known *E. coli* Tat complexes. This observed homogeneity of TatA complexes in *B. subtilis* suggests that this may be a general feature of TatA complexes in Gram-positive bacteria, and a major difference compared with Gram-negative species.

A somewhat controversial aspect of *B. subtilis* Tat studies has been the identification of a cytosolic species of TatAd that has been shown to bind the substrate PhoD [24]. This led to the suggestion that TatAd binds its substrate in the cytosol and acts as a guidance factor, targeting substrate molecules to membrane-localized TatCd by a mechanism that would be

completely different to the current *E. coli* model [25]. We therefore considered it important to test for the presence of cytosolic TatAy. We show that TatAy does indeed have a cytosolic as well as a membrane-associated localization, and the possible significance of this cytosolic TatA is discussed.

Results

TatAyCy is active in *E. coli* and able to recognize three different *E. coli* Tat signal peptides

TatAdCd has previously been shown to be active in *E. coli* and able to export fusion proteins comprising the signal peptides of TorA and DmsA linked to GFP [8]. Separate TatAdCd and TatAd complexes were characterized and shown to be very different from their *E. coli* counterparts. However, TatAdCd is an exceptional Tat system. In order to understand Gram-positive Tat systems in a more general sense, and simultaneously probe the basis for the observed strict substrate specificities of TatAdCd and TatAyCy in *B. subtilis*, we analysed the TatAyCy system in terms of substrate specificity and complex organization. A key aim was to probe the mechanism of the TatAyCy system in the light of suggestions that Gram-positive Tat systems may operate in a fundamentally different manner to those of Gram-negative organisms.

In order to directly compare the substrate specificities of the TatAdCd and TatAyCy systems, we first tested whether overexpressed TatAyCy is likewise able to form an active translocation system in *E. coli*, with the aim of analysing the abilities of the two systems to transport a range of substrates. The *tatAyCy* genes were overexpressed in an *E. coli* *tat* null (*AtatABCDE*) mutant on the pEXT22 plasmid alongside one of three heterologous Tat substrates expressed on the compatible pBAD24 plasmid. The substrates comprised green fluorescent protein (GFP) fused to the Tat signal peptides of *E. coli* AmiA, MdoD or DmsA. In addition, wild-type *E. coli* (MC4100) cells expressing the substrate were used as a positive control for export and *AtatABCDE* cells were used as a negative control. Following expression from both plasmids, cells were fractionated into periplasm (P), cytoplasm (C) and membrane (M) fractions and analysed by immunoblotting with anti-GFP serum. Figure 1A shows that AmiA–GFP is exported by wild-type (wild-type) cells, with mature-size GFP detected in the periplasmic fraction (P). No periplasmic band was observed in the *tat* null mutant strain as expected, but most of the AmiA–GFP is exported when either TatAyCy or TatAdCd is expressed in the *tat* null background, with strong

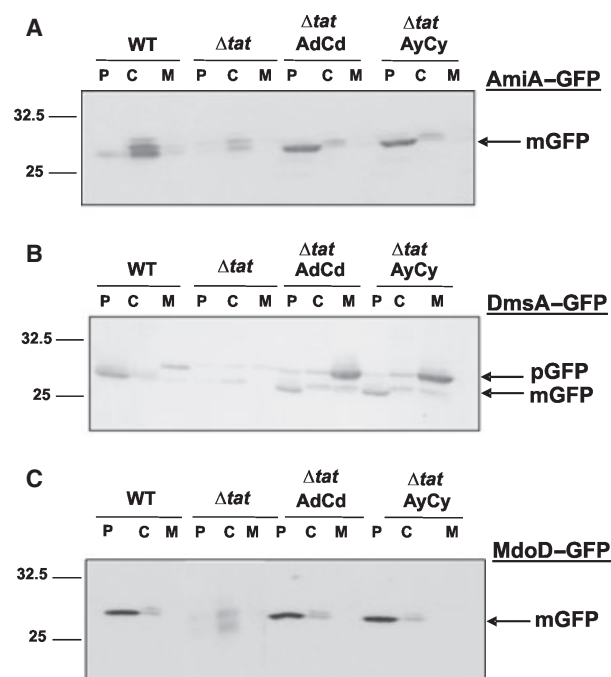


Fig. 1. Expression of *B. subtilis* TatAyCy and TatAdCd leads to export of AmiA–GFP, DmsA–GFP and MdoD–GFP in *E. coli*. Constructs comprising the (A) AmiA, (B) DmsA or (C) MdoD signal peptide (SP) linked to GFP (TatSP–GFP) were expressed from the pBAD24 plasmid in wild-type MC4100 cells, $\Delta tatABCDEF$ cells (Δtat) and $\Delta tatABCDEF$ cells expressing *B. subtilis* TatAyCy or TatAdCd (using the compatible pEXT22 plasmid). Cells were fractionated into periplasmic, cytoplasmic and membrane components (P, C, M) which were immunoblotted using specific anti-GFP serum. Mobilities of the precursor forms and mature-size GFP are indicated (pGFP, mGFP). Mobilities of molecular mass markers are given on the left (in kDa).

mature-size GFP (mGFP) signals in the periplasmic samples. Indeed, export is more efficient than with wild-type cells (where the periplasmic mature band is rather weak) but this may reflect the fact that the *tatAdCd* genes, or the *tatAyCy* genes, are overexpressed compared with wild-type cells. This demonstrates for the first time that TatAyCy is active in *E. coli*. The cytosolic fractions contain bands caused by proteolytic cleavage of the precursor protein, as observed previously (it should also be noted that this assay is not quantitative and the amount of protein detected is variable, again as described previously) [8]. Essentially the same results were obtained with the two other substrates tested; DmsA–GFP export assays are shown in Fig. 1B and MdoD–GFP assays are shown in Fig. 1C. Both substrates are exported by wild-type *E. coli* cells, and by cells overexpressing TatAyCy and TatAdCd. MdoD–GFP, in particular, is an excellent substrate for these studies with the vast majority

exported by both *B. subtilis* systems as well as by the TatABC system in wild-type *E. coli* cells. DmsA–GFP is exported more efficiently in wild-type cells than in TatAdCd- or TatAyCy-expressing cells, with a greater accumulation of precursor protein (pGFP) evident in the latter cases. The precursor protein is mostly found in the membrane fraction, in agreement with earlier studies [26] in which a Tat signal peptide–GFP fusion was found to accumulate strongly with the membrane if not exported (much of the membrane-bound GFP was incorrectly folded, suggesting a nonspecific interaction rather than a specific interaction with the translocon). Nevertheless, these cells clearly export DmsA–GFP with a clear periplasmic mature-size band apparent in both cases. No export of any substrate was observed in the *tat* null mutant strain, and most of the cytoplasmic protein is degraded as observed in a previous study involving the use of DmsA–GFP [27]. This shows that the two *B. subtilis* Tat systems recognize all three Tat signal peptides, despite exhibiting markedly different substrate specificities in *B. subtilis*.

TatAyCy is unable to transport some Tat substrates

We also tested whether TatAyCy can transport a natural *E. coli* Tat substrate, trimethylamine *N*-oxide reductase (TorA). TorA is one of the largest known Tat substrates (90 kDa), and is required for anaerobic growth on minimal trimethylamine *N*-oxide (TMAO) and glycerol media. Well-established export assays have been described for TorA transport and we have shown previously that the TatAdCd system can efficiently export this substrate when expressed in *E. coli* *tat* mutant cells [21]. Figure 2A shows a TorA export assay in which TorA activity is detected using a native polyacrylamide gel involving a methyl-viologen-based reduction that results in the clearing of gel turbidity in the presence of TMAO reductase and substrate. The left-hand panel shows wild-type *E. coli* as a positive control. A white band is clearly present in the periplasmic (P) lane, indicating export as expected. Some TorA activity is also apparent in the cytosolic (C) fraction as has been observed previously. No activity was detected in the membrane (M) fraction. As a negative control we also ran samples from the *E. coli* *tat* mutant strain (*ΔtatABCDE*), and TorA activity is exclusively cytosolic in these cells. As a second positive control we analysed cell fractions from *E. coli* *ΔtatABCDE* cells expressing TatAdCd from the pBAD24 plasmid. As described previously, TorA is exported to the periplasm with high efficiency. By contrast, the right-hand panel shows samples from *ΔtatABCDE*

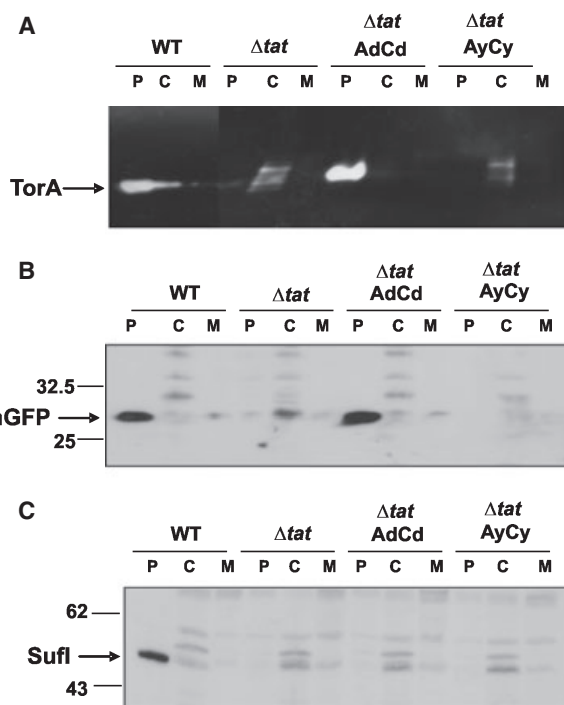


Fig. 2. TMAO reductase, TorA–GFP and SufI are not transported by TatAyCy. (A) Native polyacrylamide gel stained for TMAO reductase (TorA) activity. Membrane, cytoplasmic and periplasmic fractions (M, C and P) were prepared and analysed from wild-type MC4100 cells, $\Delta tatABCDE$ cells (Δtat) and $\Delta tatABCDE$ cells expressing *B. subtilis* TatAyCy or TatAdCd from plasmids pEXT–AyCy and pEXT–AdCd. Mobility of active TorA is indicated. (B) A construct comprising the TorA signal peptide fused to GFP (TorA–GFP) was expressed from plasmid pBAD24 in wild-type MC4100 cells, $\Delta tatABCDE$ cells (Δtat) and $\Delta tatABCDE$ cells expressing *B. subtilis* TatAyCy or TatAdCd from plasmids pEXT–AyCy and pEXT–AdCd (using the compatible pEXT22 plasmid). Cells were fractionated into periplasmic, cytoplasmic and membrane components (P, C, M) which were immunoblotted using specific anti-GFP serum. Mature size GFP is indicated (mGFP). (C) Cell fractions prepared from wild-type MC4100 cells, $\Delta tatABCDE$ cells (Δtat) and $\Delta tatABCDE$ cells expressing *B. subtilis* TatAyCy or TatAdCd from plasmids pBAyCy and pBAdCd were also immunoblotted using specific anti-SufI serum. The mobility of mature SufI is indicated and molecular mass markers are indicated on the left (in kDa).

cells expressing TatAyCy, and the data show that TorA activity is localized exclusively in the cytoplasmic fraction, with no export apparent. Thus, although both TatAdCd and TatAyCy were able to translocate the three substrates tested above, not all substrates are compatible with the TatAyCy system and a degree of substrate specificity is observed between the two pathways.

Given that TatAyCy cannot export TorA, this could be because of an inability to: (a) recognize the TorA signal peptide, or (b) handle the mature TorA protein. We addressed the first possibility by expressing the

tatAyCy genes on the pEXT22 plasmid as described above, together with a construct comprising the TorA signal peptide fused to GFP on the pBAD24 plasmid; the data are shown in Fig. 2B. In control tests, TorA–GFP is exported to the periplasm (P) in wild-type *E. coli* cells as shown previously [21]. In the *E. coli tat* mutant strain (Δtat) no band is apparent in the periplasmic fraction, again as expected. We have shown previously [21] that TatAdCd is able to efficiently translocate TorA–GFP, and as an additional positive control we expressed TatAdCd from the pEXT22 plasmid together with the pBAD–TorA–GFP construct in the *E. coli* $\Delta tatABCDE$ strain. Reproducing earlier findings, we observe mature-size GFP in the periplasmic fraction, confirming export. Finally, the right-hand panel shows that TatAyCy-expressing cells are unable to transport TorA–GFP, as no GFP is detectable in the periplasmic fraction. The TatAyCy system is thus unable to recognize the TorA signal peptide (because TatAyCy can transport GFP when other Tat signals are attached; Fig. 1). This may explain the failure to export the native TorA precursor protein, but we should point out that our data do not exclude the possibility that TatAyCy may also be incapable of handling the TorA mature protein.

We finally tested one other *E. coli* Tat substrate for export by TatAyCy. SufI is a 50-kDa *E. coli* periplasmic protein thought to play a role in cell division [28]. We have shown previously that SufI cannot be exported by the TatAdCd pathway [21], and Fig. 2C shows tests to determine whether TatAyCy can export this substrate. The left-hand panel shows fractions from *E. coli* wild-type cells, with mature-size SufI detected in the periplasmic (P) fraction. No such band was observed in the periplasm of *E. coli* $\Delta tatABCDE$ cells as was expected. The remaining panels show that neither TatAdCd nor TatAyCy (both expressed from the pBAD plasmid) are able to support export, with no SufI detectable in the periplasm. In summary, both the TatAdCd and TatAyCy pathways are active in *E. coli* and able to recognize several different *E. coli* Tat signal peptides, but not all substrates are compatible and a degree of substrate specificity is evident between the two pathways.

The TatAyCy and TatAdCd pathways recognize the same targeting determinants within signal peptides

We have previously shown, by site-directed mutagenesis of Tat signal peptides, that the *E. coli* TatABC and *B. subtilis* TatAdCd systems recognize similar targeting determinants within the signal peptides of their sub-

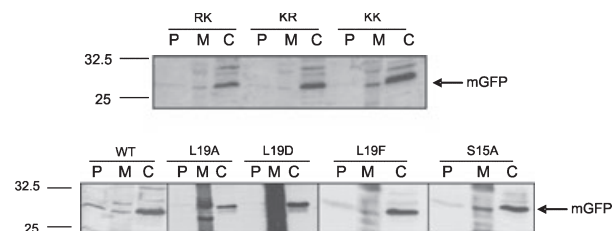


Fig. 3. Mutagenesis of the DmsA signal peptide. Constructs comprising the DmsA-signal peptide fused to GFP carrying different mutations (as indicated) were expressed from the pBAD24 plasmid along with TatAyCy expressed from the compatible pEXT22 plasmid in *E. coli* $\Delta tatABCDE$ cells. Cells were fractionated into periplasmic, cytoplasmic and membrane components (P, C, M) which were immunoblotted using specific anti-GFP serum. Mature size GFP is indicated (mGFP) and the mobilities of molecular mass markers (in kDa) are indicated on the left.

strates [8]. Within the DmsA signal peptide we found that the twin arginine motif, the -1 serine residue and the $+2$ leucine residue (with respect to the twin-arginine motif) are all important for efficient translocation by TatAdCd and *E. coli* TatABC. In order to determine if this was also true for TatAyCy we tested the ability of TatAyCy to export the DmsA–GFP fusion protein containing specific mutations within the signal peptide. We initially focused on the most conserved residues within the consensus motif, the twin arginines. Figure 3 shows that the nonmutated DmsA–GFP construct (wild-type) is exported in these cells and processed to the mature size in the periplasm (P). A considerable amount of mature-size protein is also found in the cytoplasm (C), due to nonspecific proteolysis of the signal peptide [8]. The data also show that substitution of both arginines to lysines (KK) results in a complete block in export, as no mature-sized GFP band is evident in the periplasmic sample (P). Substitution of single arginine residues to lysine (RK, and KR) results in a level of export that is so low it is barely detectable using our assay system. Only a very weak mature-size GFP band is observed in the periplasmic fraction, confirming the importance of these two residues for export. We also tested the importance of the $+2$ leucine residue (Leu19) within the consensus motif by testing for export of the DmsA–GFP fusion carrying the L19A, L19D and L19F mutations. In results similar to those obtained with TatAdCd, we find that the L19A and L19D mutations result in a complete block in export, although the L19F mutant allows a low but detectable level of translocation to occur (indicated by the presence of mature-size GFP in the periplasmic sample lane). Finally, we tested one other residue, the highly conserved -1 serine (Ser15) by replacing it with alanine. We find that this substitution

allows for only a very low level of translocation activity as indicated by a weak mature-size GFP band in the periplasmic sample. Again, this result is similar to that obtained with the TatAdCd system using the same mutated DmsA–GFP. We conclude that the TatAyCy and TatAdCd systems are not only capable of recognizing a very similar set of Tat signal peptides, but they also recognize the same conserved targeting determinants within the Tat consensus motif that are indispensable for productive protein translocation.

Several of the mutated precursor proteins associate strongly with the membrane in the absence of efficient export. It is possible that the proteins are associating with the translocon but failing to be properly translocated. However, as pointed out above, we have also observed very strong membrane-association of precursor proteins in other studies and we favour the explanation that the membrane-association is nonspecific [26].

Characterization of separate TatAyCy and TatAy complexes formed during overexpression of the *tatAyCy* genes

In order to study the TatAyCy translocase complexes, *E. coli* Δ *tatABCDE* cells expressing TatAyCy-strep (with a Strep-II tag fused to the C-terminus of TatCy) from the plasmid pBAyCys were fractionated and membranes were isolated. Total membranes were solubilized in 2% digitonin and subjected to streptactin affinity chromatography as described in Materials and methods. All column fractions were immunoblotted using antibodies to the Strep-II tag on TatCy, and to TatAy (Fig. 4, upper). Using the anti-Strep serum, a proportion of TatCy-strep was detectable in the column wash fractions, but most of the protein bound to the column. The TatCy-strep was then specifically eluted from the column across elution fractions 2–5 with a clear peak in fraction 3 (arrowed). A corresponding band was present in the same peak elution fraction in the TatAy immunoblot, indicating the presence of a TatAyCy complex. The vast majority of the TatAy protein did not bind the column and was detected in the first few column wash fractions, indicating the presence of a separate TatAy complex.

To confirm the association of TatAy and TatCy in a complex, the peak fractions from the first column were pooled and run on a second streptactin column (Fig. 4, lower). The column was washed and eluted in the same manner and the data show that the majority of both subunits co-elute in the elution fractions. This confirms the presence of a TatAyCy complex. In summary, the combined data clearly point to the presence

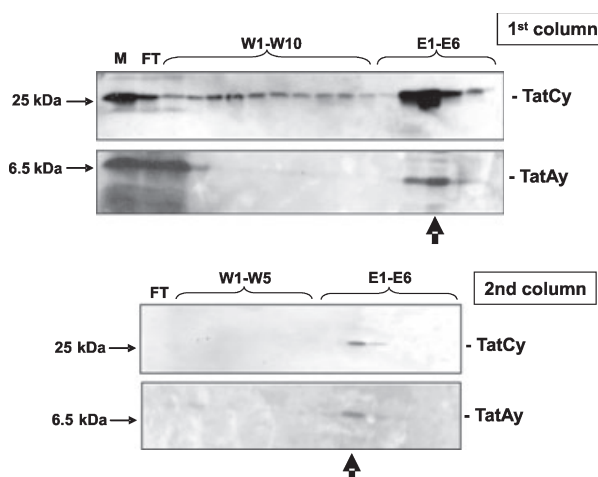


Fig. 4. Separation of distinct TatAyCy membrane-bound complexes. Membranes were prepared from Δ *tatABCDE* cells expressing *B. subtilis* TatAyCy (from plasmid pBAyCys), solubilized in digitonin and applied directly to a Streptactin affinity column as described in Materials and methods. All column fractions were immunoblotted using antibodies against the strep-II tag on TatCy and to TatAy. Peak TatCy- and TatAy-containing fractions in the elution series (E2–4) were pooled and re-run on a second column, which was processed in an identical manner. Whole membranes (M), column flow through (FT), wash fractions (W1–10) and elution fractions (E1–6) are all indicated. Mobility of TatCy-strep and TatAy are indicated on the right. Molecular mass markers are indicated on the left.

of separate TatAyCy and TatAy complexes, and the key point is that this two-complex organization is a common feature of all Tat systems analysed in this way to date [12,21].

Gel-filtration chromatography reveals a TatAyCy complex of ~ 200 kDa

Apart from the absence of a TatB component, the earlier study on the TatAdCd system revealed a major difference from the *E. coli* TatABC system in that TatAd is present as a small, highly homogeneous complex [21]. The corresponding *E. coli* TatA complex is remarkably heterogeneous, with an average size far greater than the TatAd complex, and this raises the possibility that the TatAd complex is atypical, with its restricted size distribution perhaps related to the narrow substrate specificity. To characterize a second Gram-positive Tat system in this respect, we examined the size and characteristics of both the TatAyCy and TatAy complexes.

For analysis of the isolated TatAyCy complex, the peak elution fractions from the streptactin column (see above) were pooled, concentrated and applied to a

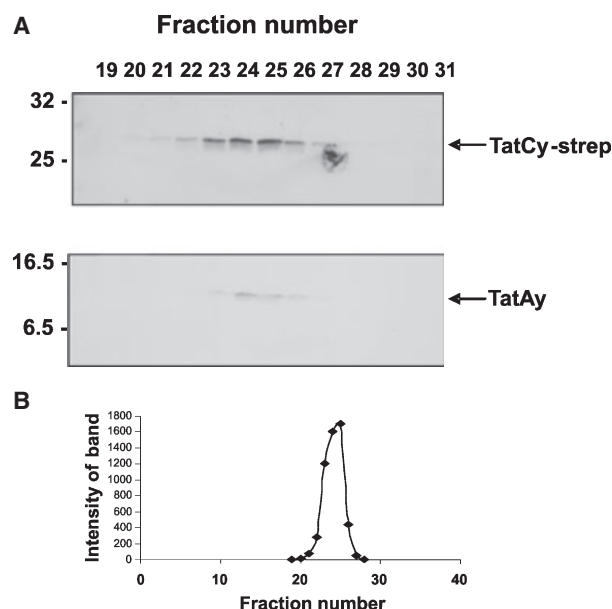


Fig. 5. Purified TatAyCy is a discrete 200 kDa complex. (A) Affinity purified TatAyCys was applied to a Superose-6 gel filtration column as described in Materials and methods. Peak elution fractions (19–31) were immunoblotted using antibodies against the strep-II tag on TatCy and to TatAy. Mobility of TatCy-strep and TatAy are shown on the right. Molecular mass markers (in kDa) are indicated on the left. (B) The TatCy immunoblot was analysed by densitometry and intensities of bands plotted against fraction number. The column was calibrated using a set of protein standards of known molecular mass, namely thyroglobin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa).

calibrated Superose-6 gel filtration column. All column elution fractions were immunoblotted using antibodies to the Strep-II tag on TatCy or to TatAy (Fig. 5A). The immunoblots show TatCy to elute in fractions 20–28 with a peak in fraction 25. A small amount of TatAy co-elutes with TatCy, confirming that these two components are present as a stable complex. Only a very small proportion of the TatAy protein present in the plasma membrane is found in this complex as shown above using affinity chromatography. This is reflected by the weakness of the band that is detectable in the TatAy immunoblot. The peak elution fractions were analysed by densitometry and band intensity was plotted against fraction number (Fig. 5B). The data show that the complex is eluting as a relatively tight peak (suggesting that TatAyCy is rather homogeneous) and calibration of the column shows the complex to be ~ 200 kDa, significantly smaller than the *E. coli* TatABC complex (600 kDa by gel-filtration chromatography) [13] and even smaller than the TatAdCd complex (350 kDa by gel-filtration chromatography) [21]. These size estimates are influenced by the size of

the detergent micelle and the true sizes of the protein complexes are likely to be smaller.

Membrane-bound TatAy complex is small and homogeneous (~ 200 kDa), whereas cytosolic TatAy forms large complexes or aggregates (~ 5 MDa)

The TatAy complex was analysed in a similar manner, but in this case we studied the complex after isolation from both the membrane and cytosol fractions. Recent work on the TatAdCd pathway of *B. subtilis* has shown that TatAd is in the cytosol as well as the plasma membrane, and the cytosolic form has been proposed to act as the initial receptor for substrates [24]. We first sought to determine whether TatAy also displays this dual localization when expressed in *E. coli*. For this purpose, we introduced plasmid pBAyCys in *E. coli* Δ tatABCDE cells and then cytosolic (C) and membrane (M) samples were analysed using specific TatAy antibodies (Fig. 6A). The data show that TatAy is indeed present in both membrane and cytosolic fractions. We also analysed the size and homogeneity of the cytosolic and membrane-associated TatAy complexes, using the Superose-6 column as above. This column has a separation range of 5 kDa to 5 MDa. We found that cytosolic TatAy eluted over fractions 8–12 with a peak in fraction 10 (Fig. 6B), which equates to a size of ~ 10 MDa, as determined from the calibration curve prepared using the markers detailed in Fig. 5. This value is above the theoretical maximum size range given for this column, which prevents us from making an accurate determination of the complex size. Nevertheless, the data demonstrate that the cytosolic TatAy complex is ~ 5 MDa (or larger). It is therefore likely that the cytosolic TatAy is forming large complexes or aggregates in the same way as cytosolic TatAd has been found to do previously [21]. A slower migrating band is also present in the immunoblot that follows the elution pattern of monomeric TatAy (indicated with *). This band may represent a dimer or trimer of cytosolic TatAy.

By contrast, membrane-localized TatAy eluted across fractions 20–30 with a peak in fraction 25 (Fig. 6C), corresponding to a size of ~ 200 kDa. Finally, immunoblots were analysed by densitometry and the intensity of the bands was plotted against the fraction number, with cytosolic TatAy indicated by filled squares and membrane-bound TatAy by open squares (Fig. 6D). The data confirm that the membrane-bound TatAy complex is much smaller than the cytosolic TatAy complex; it is also far more

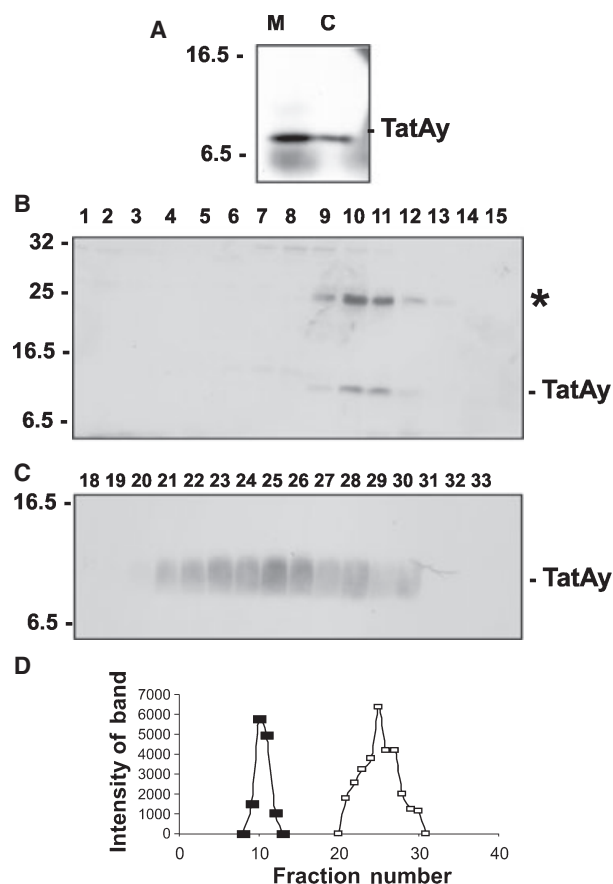


Fig. 6. TatAy forms a ~200 kDa complex within the plasma membrane and large aggregates within the cytosol. (A) Membrane (M) and cytosolic (C) fractions of *E. coli* $\Delta tatABCDE$ cells overexpressing TatAyCys were immunoblotted with anti-TatAy serum. Molecular mass markers (in kDa) are shown on the left and mobility of TatAy on the right. (B) The cytosolic fraction of *E. coli* $\Delta tatABCDE$ cells overexpressing TatAyCy was applied directly to a superpose-6 HR gel-filtration column in the absence of detergent and peak elution fractions (1–15) were immunoblotted with anti-TatAy serum. Molecular mass markers are indicated on the left. Mobility of TatAy is shown. A slower running band in lanes corresponding to the TatAy elution is indicated with an asterisk. (C) Streptactin column flow-through and wash fractions containing free TatAy complexes isolated from membranes from TatAyCy overexpressing cells were also subjected to gel-filtration chromatography and peak elution fractions (18–33) were immunoblotted with anti-TatAy serum. Mobilities of molecular mass markers (in kDa) are indicated on the left and mobility of TatAy shown on the right. (D) Immunoblots of all elution fractions of membrane-localized and cytosolic TatAy were analysed by densitometry. Intensities of the bands were plotted against fraction number. The column was calibrated using standards of known molecular mass as detailed in Fig. 5. Membrane localized TatAy is indicated with open squares and cytosolic TatAy is indicated with filled squares.

homogeneous when compared with *E. coli* TatA complexes that were isolated and run under exactly the same conditions [12,14].

Discussion

Most studies on bacterial Tat pathways have been carried out on *E. coli*, with broadly similar results obtained in studies carried out using other Gram-negative bacteria [13]. These studies have identified separate TatABC and TatA complexes in the plasma membrane, with the latter varying in size from < 100 to > 500 kDa [12,14]. Current data point to a model where, following substrate binding to the TatABC complex [29], the TatA complex is recruited to form the full translocation system.

Gram-positive organisms usually lack a TatB component, and this suggests that the TatA component is bifunctional, fulfilling the roles of both *E. coli* TatA and TatB. This has been confirmed experimentally for TatAd from *B. subtilis* [16]. However, studies on the TatAdCd system also revealed other differences, especially concerning the nature of the Tat complexes. In this study, we have sought to study the second Tat complex of *B. subtilis*, TatAyCy, to determine similarities and differences that may shed new light on the substrate specificity displayed by both complexes in their host organism.

In our previous work, gel-filtration chromatography using the detergent digitonin gave a size estimate of ~600 kDa for *E. coli* TatABC [22] but just 350 kDa for TatAdCd [21]. In this study, we show the TatAyCy complex to be even smaller with a size estimate of just 200 kDa. It thus appears that a clear difference may exist between the Tat complexes of Gram-negative and Gram-positive bacteria in terms of the size of the TatC-containing substrate-binding complex. Some of the difference may stem from the absence of a TatB component, but the complexes may well contain differing numbers of TatC-containing domains and this important question merits further attention.

The most notable characteristic of the TatAd complex is that it displays none of the heterogeneity found among *E. coli* TatA complexes [12,14]. Here, we show that the TatAy complex is both small and homogeneous with an estimated size (using gel-filtration chromatography) of ~200 kDa, which is again even smaller than the TatAd complex (estimated to be ~270 kDa under the same conditions) [21]. The key point is that the TatAy complex, like the TatAd complex, is relatively homogeneous. This provides the first indication of a major general difference between TatA complexes of Gram-positive and Gram-negative bacteria. In this context, it is interesting that the *B. subtilis* Tat systems are capable of transporting a variety of substrates with very different sizes. This finding

suggests that there is no strict correlation between TatA heterogeneity and substrate sizes.

Our data also have relevance for the biological roles of the TatAdCd and TatAyCy systems in *B. subtilis*. Here, we have shown that both the TatAdCd and TatAyCy systems are able to recognize and transport a wide variety of heterologous signal peptides, all of which differ widely in primary sequence. Clearly, the TatAdCd system is not predisposed to interact with only the PhoD signal peptide. Moreover, the mutagenesis studies strongly suggest that the signal peptide determinants shown to be recognized by TatAdCd [8] are equally important for productive interaction with the TatAyCy system. This raises the question of why two distinct Tat systems are present in *B. subtilis*, and this question remains open. One possibility would be that the TatAdCd system provides additional capacity for Tat-dependent protein export under conditions of phosphate starvation that lead to a massive induction of PhoD synthesis.

A controversial aspect of studies into the TatAdCd system of *B. subtilis* was the identification of a soluble substrate-binding species of TatAd in the cytoplasm [24]. This led to the idea that the substrate first interacts with cytosolic TatAd before targeting to the membrane-localized TatCd component [25]. This would imply that completely different mechanisms might be operating in *B. subtilis* and *E. coli*. We recently found that the TatAdCd pathway was active in an *E. coli* background and able to translocate the *E. coli* Tat substrate TMAO reductase (TorA) [21]. In *E. coli*, TorA has its own dedicated cytosolic chaperone TorD, which binds strongly to its signal peptide prior to its recognition by the membrane localized TatABC substrate-binding complex [30]. The fact that TatAdCd can translocate TorA in *E. coli* suggested that this Tat system is operating in a manner more closely resembling the *E. coli* model. We further found that cytosolic TatAd, present following overexpression of TatAdCd in *E. coli*, was forming large complexes or aggregates for which a possible role in the translocation process is difficult to assess unambiguously [21]. We therefore considered it important to test for the presence of cytosolic TatAy. We did indeed find that alongside its membrane localization, TatAy was present as a soluble species in the cytosol. We also found that, like cytosolic TatAd, TatAy forms very large complexes or aggregates. The functional significance of this pool of cytosolic TatA is not clear. A recent study has found that *E. coli* TatA can also be found in the cytoplasm where it forms large homo-oligomeric complexes in tube-like structures [31]. The presence of large soluble TatA complexes in *E. coli* suggests that the Tat

systems of Gram-positive and Gram-negative bacteria may be more similar than previously thought. This fits well with our observation that the *E. coli* and *B. subtilis* Tat systems are capable of translocating a similar set of substrates, but makes the differences we observe between the membrane-localized Tat complexes of *E. coli* and *B. subtilis* all the more intriguing. However, the ability of *E. coli* to export such a range of substrates, when coexpressing either the native Tat system or either *B. subtilis* Tat system, provides a powerful tool to investigate both the structures and functions of the different Tat complexes.

Materials and methods

Bacterial strains, plasmids and growth conditions

All strains and plasmids used are listed in Table 1. *E. coli* MC4100 [32] was used as the parental strain. The $\Delta tatA-BCDE$ strain [11] has been described previously. Arabinose-resistant derivatives were used as described previously. *E. coli* was grown aerobically in Luria–Bertani broth at 37 °C. *E. coli* was grown anaerobically in Luria–Bertani broth supplemented with 0.5% glycerol, 0.5% TMAO and 1 μ M ammonium molybdate. Media were supplemented with ampicillin to a final concentration of 100 μ g·mL⁻¹, kanamycin to 50 μ g·mL⁻¹, arabinose to 0.5 mM and isopropyl thio- β -D-galactoside to 5 mM when required. *B. subtilis* was grown in trypton/yeast extract medium, consisting of Bactotryptone (1%; w/v), Bacto yeast extract (0.5%; w/v) and NaCl (1%; w/v), unless indicated otherwise. Media were supplemented with kanamycin (20 μ g·mL⁻¹), chloramphenicol (5 μ g·mL⁻¹) and/or spectinomycin (100 μ g·mL⁻¹).

DNA techniques

For arabinose inducible overproduction of the *B. subtilis* *tatAyCy* operon with a C-terminal strep-II tag attached to the TatC component, plasmid pBAyCys was constructed as follows. The *tatAyCy* operon was amplified from *B. subtilis* 168 chromosomal DNA with primers RTEAyF (5'-CGCGTCTCGCATGCCGATCGGTCCTGGAAGCCTTGCTG-3') and JJystrep02 (5'-ATATTCTAGATTATTTTTCAAACTGTGGGTGCGACCAATTCGATTGCCAGAAGACACGTCCCG-3'). RTEAyF was designed as such that restriction of the generated *tatAyCy*–strep PCR-amplified fragment with dovetail enzyme *Esp3I* would create a *NcoI* overhang, to ensure direct cloning in the vector pBAD24. JJystrep02 was constructed as such that a C-terminal strep-II tag (underlined) would be directly attached to *tatCy* during the PCR amplification. pBAyCys was constructed by ligating an *Esp3I*- and *XbaI*-cleaved PCR-amplified fragment of *tatAyCy* into *NcoI*- and

Table 1. Bacterial strains and plasmids used in this study.

Plasmids	Relevant properties	Ref./source
pBAD-ABC	pBAD24 derivative containing the <i>E. coli</i> <i>tatABC</i> operon; Amp ^r	[33]
pBADcd	pBAD24 derivative containing the <i>B. subtilis</i> <i>tatAdCd</i> operon; Amp ^r	[21]
pBAyCy	pBAD24 derivative containing the <i>B. subtilis</i> <i>tatAyCy</i> operon; Amp ^r	This study
pBAD-DmsA-GFP	pBAD24 derivative containing DmsA-GFP Amp ^r	[27]
pBAD-DmsA-GFP L19A	pBAD24 derivative containing DmsA-GFP Amp ^r	[8]
pBAD-DmsA-GFP L19D	pBAD24 derivative containing DmsA-GFP Amp ^r	[8]
pBAD-DmsA-GFP L19F	pBAD24 derivative containing DmsA-GFP Amp ^r	[8]
pBAD-DmsA-GFP S15A	pBAD24 derivative containing DmsA-GFP Amp ^r	[8]
pBAD-DmsA-GFP RK	pBAD24 derivative containing DmsA-GFP Amp ^r	This study
pBAD-DmsA-GFP KR	pBAD24 derivative containing DmsA-GFP Amp ^r	This study
pBAD-DmsA-GFP KK	pBAD24 derivative containing DmsA-GFP Amp ^r	This study
pJDT1	pBAD24 derivative containing TorA-GFP Amp ^r	[35]
pBAD-AmiA-GFP	pBAD24 derivative containing AmiA-GFP Amp ^r	This study
pBAD-MdoD-GFP	pBAD24 derivative containing MdoD-GFP Amp ^r	This study
pEXT-AdCd	pEXT22 derivative containing the <i>B. subtilis</i> <i>tatAdCd</i> operon; Kan ^r	[21]
pEXT-AyCy	pEXT22 derivative containing the <i>B. subtilis</i> <i>tatAyCy</i> operon	
Kan ^r	This study	
Strains		
<i>E. coli</i> MC4100	F ⁻ Δ lacU169 <i>araD</i> 139 <i>rpsL</i> 150 <i>relA</i> 1 <i>ptsF</i> <i>rbs</i> <i>flbB</i> 5301	[31]
MC4100 Δ tatABCDE	<i>tat</i> deletion strain	[11]
<i>B. subtilis</i> 168	<i>trpC</i> 2	[36]

*Xba*I-cleaved pBAD24. For isopropyl thio- β -D-galactoside-inducible overproduction of *B. subtilis* TatAyCy, *tatAyCy-strep* was cut out of pBAyCys with *Nhe*I and *Xba*I and ligated into *Nhe*I/*Xba*I-cut pEXT22 to construct pEXT-AyCy.

For construction of pBAD-AmiA-GFP and MdoD-GFP the signal sequences for the two Tat substrates AmiA and MdoD were amplified by PCR from *E. coli* genomic DNA using the primers PCR_AmiA_EcoRI_for (GGCCGAATTCACCATTTATGAGCACTTTTA) and PCR_AmiA_EcoRI_rev (GGCCGAATTCGCTGTGTCCGTTGCTGGTT) for AmiA, and PCR_MdoD_EcoRI_for (GGCCGAATTCACCATTTATGGATCGTAGAC) and PCR_MdoD_EcoRI_rev (GGCCGAATTCGTCAAAACGCTGGGTTTGC) for MdoD. The PCR products were cut with *Eco*RI and then gel-purified. The expression vector pBAD24 containing *dmsA-GFP* was cut with *Eco*RI to release the DmsA signal sequence and then dephosphorylated after which the two PCR products *amiA* and *mdoD* were ligated into the vector (T4 Ligase; New England Biolabs, Hitchin, UK). The orientation of the two inserts was confirmed by sequencing.

Mutagenesis of the DmsA-GFP signal peptide was performed by site-directed mutagenesis (Qiagen, Crawley, UK). Primers used were: KRdmsAF (CGGTATTGGCTGCTGAGGTGAGTAAACGTGGTTTGG) and KRd-

msAR (CCAAACCACGTTTACTCACCTCAGCAGCCAATACCG) for KR mutation; RKdmsAF (GCTGCTGAGGTGAGTCGCAAAGGTTTGGTAAAAACG) and RKdmsAR (CGTTTTTACCAAACCTTTGCGACTCACCTCAGCAGC) for RK mutation; and KRtoKKdmsAF (GCTGAGGTGAGTAAAAAGGTTTGGTAAAAACGACAGCG) and KRtoKKdmsAR (CGCTGTCGTTTACCAAACCTTTTTACTCACCTCAGC) for KK mutation.

SDS/PAGE and western blotting

Proteins were separated using SDS/PAGE and immunoblotted using specific antibodies to TatAy and goat anti-(rabbit IgG) horseradish peroxidase (HRP) conjugate. The *Strep*-tag II on TatCy was detected directly using a strept-actin-HRP conjugate (Institut für Bioanalytik). Sufl, a Tat-dependent substrate of *E. coli*, was visualized using specific antibodies (kindly provided by T. Palmer). GFP was detected using a specific anti-GFP serum (Promega, Madison, WI, USA) followed by goat anti-(rabbit IgG) HRP conjugate. An ECL detection kit (Amersham Pharmacia Biotech, Little Chalfont, UK) was used to visualize the proteins.

TMAO reductase activity and TatPre-GFP assays

TMAO reductase activity assay was performed as described previously [33,34]. *E. coli* cells were grown anaerobically until mid-exponential growth phase prior to fractionation into periplasmic, cytoplasmic and membrane fractions. Cell fractions were loaded and separated on a 10% native polyacrylamide gel that was subsequently assayed for TMAO reductase activity as described previously. Pre-GFP export assays: a construct comprising either the TorA [35], DmsA [27], MdoD or AmiA signal peptide linked to GFP was expressed using the pBAD24 plasmid as previously described. For these experiments, TatAyCy was expressed from the compatible pEXT22 vector. Following expression from both plasmids, cell fractions were prepared as described above and immunoblotted using anti-GFP serum (Living Colors, Clontech, Mountain View, CA, USA).

Expression and purification of the TatAyCy complex and TatAy complex

E. coli Δ tatABCDE cells containing plasmid pBAyCys were grown aerobically to mid-exponential phase with induction of tatAyCy on plasmid pBAyCys using 0.5 mM arabinose. Cells were fractionated into membrane and cytosolic components as described previously, and the membranes were solubilized in 2% digitonin [33]. Solubilized membranes were incubated with 2 μ g mL⁻¹ avidin to block any biotin-containing proteins before application to an equilibrated 4 mL Streptactin affinity column (Institut für Bioanalytik). The column was washed with 10 column volumes of equilibration buffer containing Tris/HCl pH 8.0, 2% glycerol, 150 mM NaCl and 0.1% digitonin. Bound protein was eluted from the column in 6 \times 2.0 mL fractions using the same buffer as above but containing 3 mM desthiobiotin (Sigma, Poole, UK). Elution fractions were pooled and diluted 50-fold in equilibration buffer to reduce the concentration of desthiobiotin in the eluted samples before application to a second 4 mL Streptactin affinity column. This time the column was washed with five column vol of equilibration buffer and eluted in 6 \times 2.0 mL fractions using the elution buffer described above. For gel-filtration experiments affinity-purified TatAyCy was concentrated to 250 μ L using Vivaspins-4 centrifugal concentrators (molecular mass cut-off 10 000; Vivascience, Westford, MA, USA). The concentrated sample was loaded onto a Superose-6HR gel filtration column (Amersham Biosciences) and was eluted with the equilibration buffer described above [33].

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